보건학석사 학위논문

# *In Vitro* Inhibitory Effect by Korean Intestinal Microbial Isolates on *Clostridium difficile*

한국인 장내유래 세균을 대상으로 한

*Clostridium difficile*의 저해 효과 연구

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# Abstract

# *In Vitro* Inhibitory Effect by Korean Intestinal Microbial Isolates on *Clostridium difficile*

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*Clostridium difficile* infection (CDI), one of the major healthcare-associated infections (HAIs), is associated strongly with the administration of antibiotics, causing alteration of intestinal microbiota. Nevertheless, mechanistic understand and key species resist colonization of *C. difficile* are still to be elucidated. Several studies reported that the relationship between microbial metabolites and CDI resistance. Secondary bile acids, entirely produced by microbial metabolism, were previously reported as inhibitor of germination and vegetative cell growth of *C. difficile*, whereas primary bile acids, particularly taurocholic acid (TA) derivatives, were commonly used as germinant to culture *C. difficile* in laboratory since 1974. Short chain fatty acids (SCFAs), especially butyrate, have been thought to be important due to their beneficial features, such as anti-inflammatory effect. In this paper, some of Korean

intestinal isolates were evaluated their inhibitory activity *in vitro*. First, inhibitory effects on *C. difficile* vegetative cell growth were determined. The neutralized bacterial supernatants were added to *C. difficile* culture media, and the growths were measured by optical density. For known bile acid metabolizing bacteria, bile mixture was added in bacterial culture media to assess influence of bile acid metabolizing ability to inhibitory effect. Second, the preventive effects of supernatants were assessed via cytotoxicity test. Human colon cell line Caco2 and HT-29 were challenged with the toxic-containing *C. difficile* culture supernatant, with the commensal bacterial supernatants. Briefly, among tested bacteria isolates, isolates of *Lactobacillus* shows biggest reduction of *C. difficile* growth without bile acid. However, these results were reversed when they cultured with bile mixture, except *L. plantarum*. *C. scindens* showed inhibitory effects, especially when they were cultured with bile acid mixture. Although differences between species and strain were existed, *Bacteroides caccae* isolates were not effective, but *B. ovatus* isolates showed effectiveness when they were cultured in bile mixture containing medium. The butyrate-producing bacteria, *F. prausnitzii* and *R. intestinalis* did not show significant growth inhibition or cytotoxicity resistance. These results imply that commensal bacteria could prevent or promote *C. difficile* growth, depend on their bile acid metabolizing ability.

Key words: CDI, Clostridium difficile, Microbiome, bile acid, butyrate

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# I. Introduction

*Clostridium difficile*, a spore-forming gram positive anaerobe, is one of the most common pathogen causing healthcare-associated infections (HAIs). The clinical symptoms are various, range from mild diarrhea to fulminant colitis and death. These symptoms are resulting from two major virulence factors, homologous protein toxin A (TcdA) and toxin B (TcdB). Both toxins enter the intestinal cell via endocytosis, and irreversibly inactivate Rho GTPases by monoglucosylation resulting in cytoskeletal disruption and loss of tight junctions.

In the USA, *C. difficile* was responsible for 12 % of HAIs (1). The US Centers for Disease Control and Prevention categorized *C. difficile* as urgent threats for public health (2). In addition, the prevalence rate and the cost of *C. difficile* infection (CDI) in Korea is also increasing (3, 4). Prolonged antibiotic exposure and disruption of gut microbiota during hospitalization are thought to be major risk factors for CDI. However, occurrence of community-acquired cases for healthier young adults is rising (5). Among them, more than 30% of cases do not have typical risk factors like antibiotics and hospitalization (6). These epidemiological changes and outbreaks of hyper-virulence strain make CDI more serious concern.

There are sufficient evidences that the gut microbiota perform crucial role in host's resistance for CDI. FMT (fecal microbiota transplantation), a procedure that restore balance and diversity of gut microbiota by implanting microbiota from healthy donor, has been shown better effectiveness for treating CDI than conventional antibiotics therapy, especially for the recurrent cases. Even though their good prognosis, FMT has some limitations like potential risk for another infection, difficulties to recruit donor. Therefore, many efforts to treat CDI with defined

bacterial mixtures or single bacterial species, so-called bacteriotherapy. However, exactly which commensal bacteria have that feature or how they act is not clearly elucidated yet.

Several studies reported that the relationship between microbial metabolites and CDI resistance. Secondary bile acids, entirely produced by microbial metabolism, were previously reported as inhibitors of germination and vegetative cell growth of *C. difficile*, whereas primary bile acids, particularly taurocholic acid (TA) derivatives, were commonly used as germinant to culture *C. difficile* in laboratory since 1974. Short chain fatty acids (SCFAs), especially butyrate, have been thought to be important due to their beneficial features, such as anti-inflammatory effect. Otherwise, some metabolites such as succinate or sialic acid could promote *C. difficile* colonization (7, 8).

In this paper, we conducted *in vitro* evaluation of Korean intestinal bacterial isolates, particularly theirs growth inhibitory activities and cytotoxic preventive activities. Table.1 summarizes the features and references of tested bacterial species. Along with *C. scindens* KCTC5591 (ATCC35704), already showed preventive effect for CDI *in vivo* (9), some commercial strains were included for a comparison.

# II. Materials and Methods

## 1. Strains and culture conditions

*C. difficile* strain ATCC 43255 (VPI 10463) was purchased from the American Type Culture Collection (ATCC), and *C. difficile* KCTC 5009 (ATCC 9689), *Lactobacillus plantarum subsp. plantarum* KCTC 3018, *L. casei* KCTC 3019 and *C. scindens* KCTC 5591 were purchased from the the Korean Collection for Type Cultures (KCTC, Korea). *Bacteroides caccae* SNUG10313, SNUG30039, SNUG30273, SNUG30378, *B. ovatus* SNUG40408, SNUG40239, *C. scindens* SNUG40297, SNUG40402, *Faecalibacterium prausnitzii* SNUG30079, SNUG30092, SNUG30106, SNUG30120, SNUG30093, SNUG30090, *L. plantarum subsp. plantarum* SNUG10271, *L. rhamnosus* SNUG50057, SNUG50070, *Roseburia intestinalis* SNUG30016 were isolated previously in this laboratory.

*C. difficile* strains were cultured in BHIS medium (brain heart infusion medium containing 0.5% yeast extract, 0.1% cellobiose, 0.1% maltose and 0.05% L-cystein). *Bacteroides caccae* and *B. ovatus* isolates were cultured in BHI(brain heart infusion) supplemented with 5% FBS(fetal bovine serum) or Blood Agar ( TSA with 5% sheep blood). For *Lactobacillus* spp., MRS medium was used. C. scindens isolates were cultured in GAM medium, and *Faecalibacterium prausnitzii* and *Roseburia intestinalis* were cultured in YBHI medium(brain heart infusion medium containing 0.5% yeast extract). For cytotoxicity assay, *C. difficile* VPI 10463 was cultured in TY broth (10) (3% [wt/vol] Bacto tryptose, 2% [wt/vol] yeast extract and 0.05% [wt/vol] L-cycteine). All bacterial cultures were incubated in an anaerobic system (an anaerobic jar with gas pack) at 37℃.

**Table 1**. bacterial strains used in this study

| Strains | Relevant features |
|---|---|
| ***Bacteroides caccae*** | Increased abundance in post-FMT (21)<br>Nutrition competition (sialic acid metabolilsm) (8, 22)<br>Fecal recovery reduction in CDI patients (32) |
| ***Bacteroides ovatus*** | Increased abundance in post-FMT (21)<br>Nutrition competition (sialic acid metabolilsm) (8, 22)<br>Five patient were treated with a mixture containing *B. ovatus* (31) |
| ***Clostridium scindens*** | *C. difficile* infection resistance in *in vivo* test (9)<br>Bile acid metabolism (7a-dehydroxylation) (23)<br>Nutrition competition (sialic acid metabolilsm) (8, 22) |
| ***Faecalibacterium prausnitzii*** | Decreased abundance in post-antibiotics group (24, 25)<br>Nutrition competition (sialic acid metabolilsm) (8, 22) |
| ***Lactobacillus plantarum*** | Inhibit *C. difficile* growth (*in vitro*) (26)<br>Nutrition competition (sialic acid metabolilsm) (8, 22)<br>Strengthen epithelial tight junction (27) |
| ***Lactobacillus casei*** | Inhibit *C. difficile* growth (*in vitro*) (26)<br>Significant efficacy in RCT (33)<br>decresing *C. difficile* induced IL-8 production (28) |
| ***Lactobacillus rhamnosus*** | Inhibit *C. difficile* growth (*in vitro*) (26)<br>Decresing *C. difficile* induced IL-8 production (28) |
| ***Roseburia intestinalis*** | Increased abundance in post-FMT (21)<br>Butyrate-producing bacteria (29, 30) |

## 2. Preparation of bacterial supernatant

Bacterial strains were inoculated at 1:100 into fresh medium and incubated at 37℃. To obtain a cell-free supernatant, harvested 24 h cultures were centrifuged (5000 g for 5 min) and filter-sterilized (0.22 μm, Millipore). Supernatants were stored at -20℃ until used.

## 3. PCR assay of *bai*CD

Colony PCR was performed for detection of *bai*CD gene as described previously (42)

## 4. Vegetative cell growth inhibition

*C. difficile* ATCC 43255 and KCTC 5009 were cultured in BHIS broth anaerobically before stationary phase, adjusted to the same OD at 600 nm in order to standardize cell density. Then they were inoculated in broth at 1:100 into 200 μL of 1:1 mixtures of fresh medium and bacterial supernatants. Vegetative cell growths were assessed at 24 h via OD600. The blank used for standardization consisted of a mixture of 100μL each of fresh media and BHIS broth. As a control, 100 μL of broth media was added to 100 μL of *C. difficile* cell suspension, followed by incubation as indicated above.

## 5. Cytotoxicity assay

Cytotoxicity was monitored as described previously (11, 12). *C. difficile* VPI 10463 was grown in 10 ml TY broth, and 24 h supernatant was harvested. Four-fold serial dilutions were made in PBS. HT29 and Caco2 cells were grown in RPMI(supplemented with 10% v/v FBS, L-glutamine (300mg/L), 25mM HEPES and 25mM NaHCO3) and MEM(supplemented with 20% v/v FBS, 1% v/v penicillin-streptomycin), respectively. Cells were seeded for a total cell density $2 \times 10^4$ cells per well into 96-well plates and incubated at 37℃ in 5% CO2. After 48 h, the cell culture medium was removed and replaced with 60 μl of fresh cell culture media. To test cytotoxicity inhibition, HT29 and Caco2 were pre-incubated with equal volume (20 μl) of bacterial supernatants for 1 h prior to addition of toxin dilution (20 μl). Cytotoxicity was assessed after 24 h of incubation.

## 6. Agar spot test

Overnight cultures of isolates to be tested for inhibition ability spotted onto the surface of agar plates (appropriate media for each species) and pre-incubated. After colony formation, plates were overlaid with 7 ml of soft RCM agar (0.75% agar) inoculated with 5 μl of overnight cultures of *C. difficile* KCTC 5009. After 30 h and 60 h incubation at 37 ℃ in anaerobic condition, the zones of inhibition were observed. Results were recorded as positive if the clear zone is greater than or equals to 1 mm from the border of the colonies of the isolates.

## 7. Quantitation of SCFAs

SCFAs concentration in bacterial supernatant was measured by gas chromatography as previously described (13). GC-FID analysis was carried out using an Agilent 7890A GC instrument equipped with a flame ionization detector (FID) and a 30 m × 0.25 mm Nukol column (0.25 μm film thickness; from Supelco; N2 carrier gas). The oven temperature was set to 170 ℃, and the FID and injection port temperatures were set to 225℃.

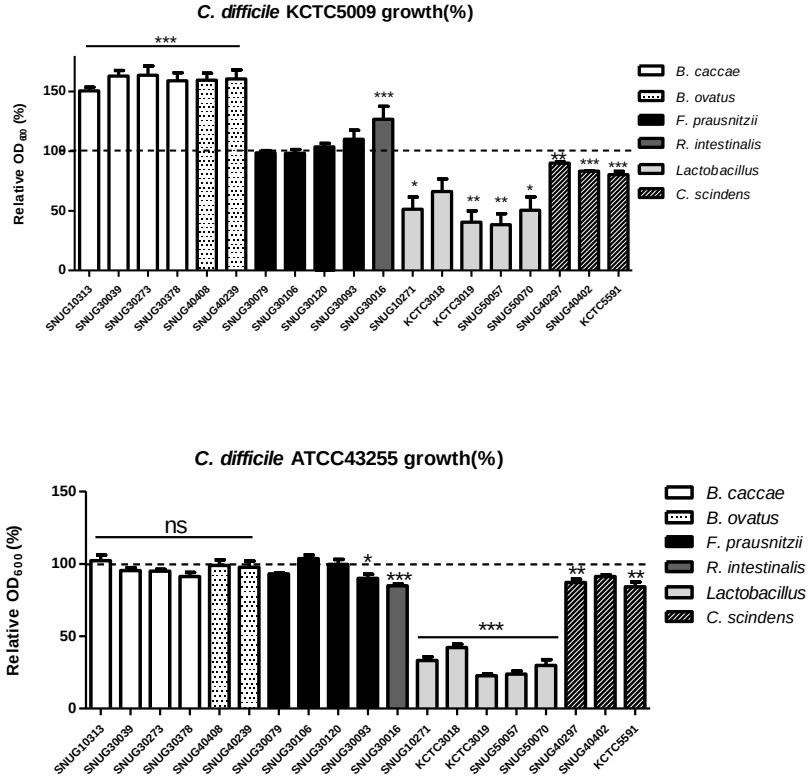
A SCFAs mix containing 40 mM of acetic and 10 mM of propionic, isobutyric, butyric, isovaleric, and valeric acids in sterile water was prepared as standard (Sigma-Aldrich). 1% 2-methyl pentanoic acid (Sigma-Aldrich) was used as internal standard.

For GC-FID analysis, 500 μl of Bacterial supernatants were mixed with 25 μl of 95% sulfuric acid (vortex for 5 min). Then samples were centrifuged (14,000 g for 5 min), and 400 μl of the supernatants were transferred into new tube with 40 μl of internal standard and 400 μl of ethyl ether. After 1 min of vortex, samples centrifuged (14,000 g for 5 min), 200 μl of the upper ether layer was collected for analysis.

# III. Results

## 1. Effects of bacterial supernatants to *C. difficile* growth

We conducted *C. difficile* vegetative cell growth test with bacterial supernatant. Two *C. difficile* strain ATCC 43255 and KCTC 5009 are inoculated in BHIS broth media with bacterial supernatant (BHIS:supernatant = 1:1). Results were measured by $OD_{600}$ at 24 h after incubation and normalized relative to each control, using fresh media for each test isolates instead of supernatants. Because *C. difficile* strains are acid-susceptible (Figure 2), bacterial supernatants were neutralized. Among tested intestinal isolates, *L. plantarum, L. casei and L. rhamnosus* significantly decrease *C. difficile* growth ($p<0.001$). On the other hand, *B. caccae* and *B. ovatus* shows significantly increase *C. difficile* KCTC 5009 growth. The mean growth of *C. difficile* is slightly decreased due to the supernatant of *C. scindens* isolates. The supernatant of *R. intestinalis* showed inhibition for ATCC 43255, but showed opposite activity to KCTC 5009 (Figure 1). This opposite pattern was observed in *F. prausnitzii* isolates, but its influences were not significant.




## Figure 1. growth of *C. difficile* in culture media containing the fraction of supernatants of gut isolates

*C. difficile* growth inhibition of bacterial supernatants. A) *C. difficile* KCTC 5009 growth OD; B) *C. difficile* ATCC 43255 growth. $OD_{600}$ values were measured by microplate reader after 24 h incubation at 37 °C in anaerobic condition. Supernatants were neutralized with NaOH or HCl to pH 6.7 ± 0.2. Triplicated results are expressed as the mean (%) ± SEM compared to the each control. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Bonferroni test. (ns: not significant; *p<0.05, **p<0.01, ***p<0.001)

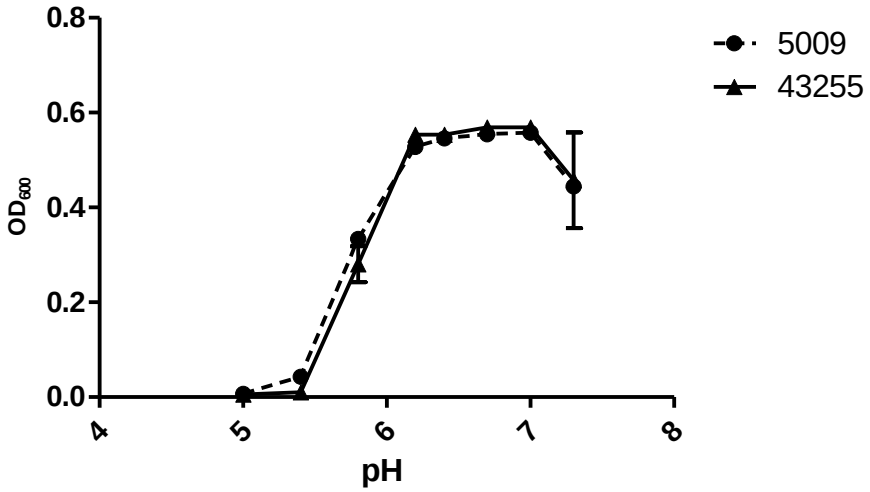



**Figure 2. *C. difficile* growth under various pH conditions**

Two *C. difficile* strains were inoculated in buffered BHIS (10% MES buffer) broth. $OD_{600}$ values were measured by microplate reader at the stationary phase. Duplicated results are expressed as the mean (%) ± SEM compared to the each control.

## 2. Some of isolates of *B. caccae* and *B. ovatus* shows inhibitory effect to *C. difficle* KCTC 5009 when assessed by agar spot assay

*C. difficile* KCTC 5009 growth with supernatants of *Bacteroides* were different with those of *C. difficile* ATCC 43255. KCTC 5009 growth was elevated compare to control when it was treated with supernatants of *Bacteroides* ($p<0.001$). To figure out whether *Bacteroides* promote *C.diffcile* growth or not, inhibitory effect of these isolates were assessed again by different method. We were tested these isolates with agar spot assay, basically followed Chapman's methods with minor modification (46). Test isolates were grown on inter-surface of two different agar media (lower is BHI+5%FBS for *bacteroides*, and upper soft agar is RCM for *C. difficile*), and *C. difficile* KCTC 5009 were grown on entire upper layer. In comparison with test conducted in broth media, some isolates shows inhibitory effect on KCTC 5009, even though inhibition zone were not very clear. Any promotive effect was not observed in this method.

**Table 2**. Agar spot test result

| Isolates | Time | |
|---|---|---|
| | 30 h | 60 h |
| *Bacteroides caccae* | | |
| SNUG10313 | - | - |
| SNUG30039 | - | - |
| SNUG30273 | + | + |
| SNUG30378 | + | - |
| *Bacteroides ovatus* | | |
| SNUG40408 | + | + |
| SNUG40239 | + | + |

\+ *C. difficile* KCTC5009 was inhibited

\- *C. difficile* KCTC5009 was not inhibited

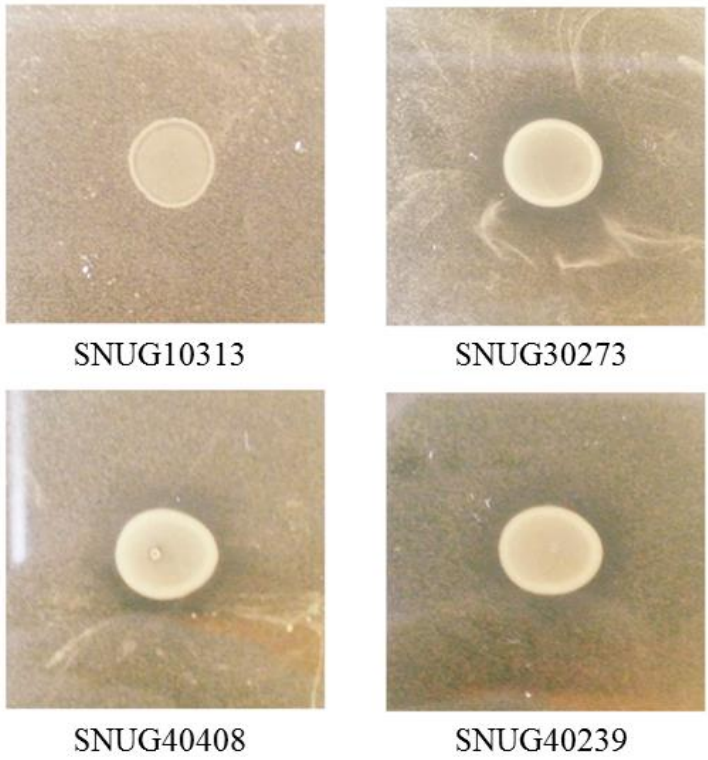



**Figure 3. Images of agar spot assay**

Images of agar spot assay. Inhibition zone of *C. difficile* KCTC5009 by *B. caccae and B. ovatus* after 60 h incubation. Test colonies were preincubated 2 days before *C. difficile* inoculation. Results were recorded as positive if the clear zone is greater than or equals to 1 mm from the border of the colonies.

### 3. Supernatants of *Lactobacillus*, *B. caccae*, *B. ovatus* and *C. scindens* inhibit *C. difficile* growth when these isolates are cultured with bile mixture

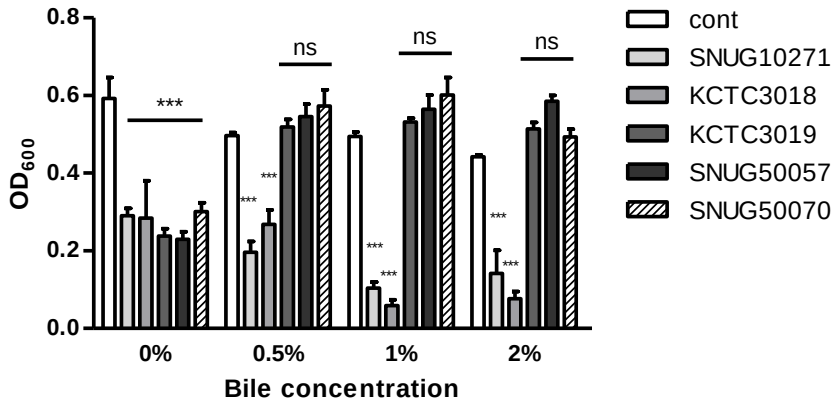
Since bile acid metabolism of intestinal bacteria is known as one of crucial factors of host resistance to CDI, bile acid metabolizing species were thought to be effective. In order to find out which isolate could utilize bile acid and thus hinders *C. difficile* growth, isolates were grown in broth media containing bile acid mixture (Sigma-Aldrich) for 24 h, then supernatants were harvested and filter-sterilized. Because the typical concentration of bile salts in the small intestine ranges from 0.2 to 2% (43, 44), bile mixture concentrations were determined same as 2% or below than 2%. Bile acid containing supernatants and bile acid-absent BHIS were applied at ratio 1:1, result in divided concentration for *C. difficile* (2% to 1%).

Some of tested *Lactobacillus* lost their effectivity. Furthermore, *L. rhamnosus* SNUG50057 made an increase of growth OD of *C. difficile* KCTC 5009. However, *L. casei* SNUG10271 and KCTC3018 maintain effectiveness for *C. difficile* KCTC5009 ($p<0.001$), and decrease growth of *C. difficile* ATCC43255 although this effect was not significant (Figure 4).

*C. scindens* showed potent reduction against *C. difficile* KCTC5009 at 2% bile acid mixture. However, *C. difficile* ATCC43255 growth OD was less significantly reduced at 2% bile mixture ($p<0.05$), due to great reduction of control result (Figure 5).

*B. caccae* has no significant inhibitory effect at any concentrations of bile mixture, except SNUG30039. *B. ovatus* shows significant effect, particularly SNUG40239 inhibit *C. difficile* ATCC43255 growth greatly ($p<0.01$). However, when they cultured at 2% bile mixture, none of them shows significant results.

**A: *C. difficile* KCTC 5009**




**B: *C. difficile* ATCC 43255**

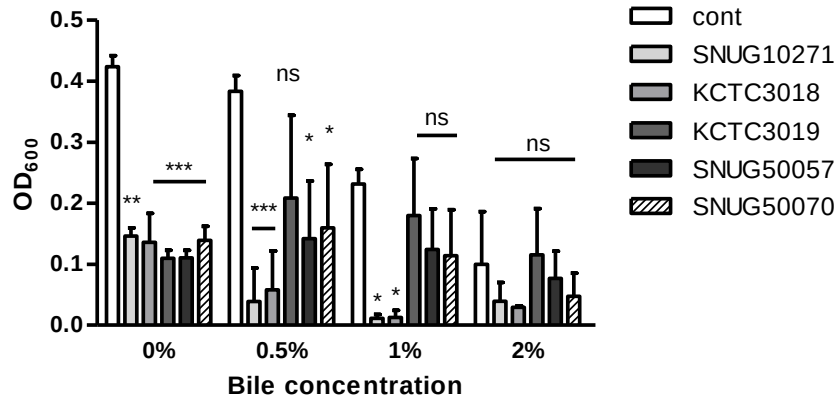
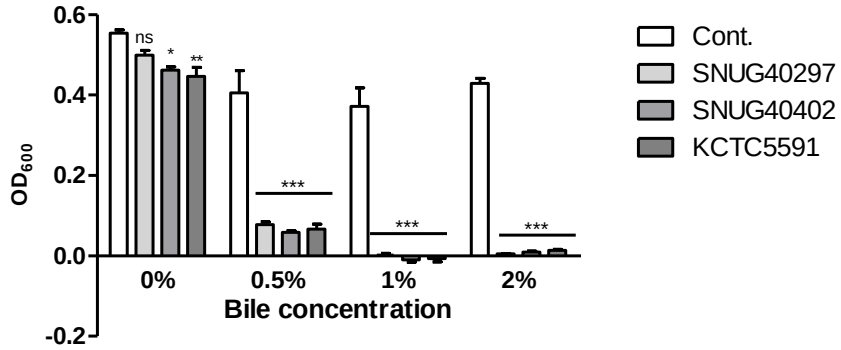



**Figure 4. Effects of bile mixture in culture supernatants of *Lactobacillus* isolates on *C. difficile* growth**

Inhibitory effect of C. scindens when it was cultured in bile mixture containing media. A) *C. difficile* KCTC 5009 growth OD; B) *C. difficile* ATCC 43255 growth. OD600 values were measured by microplate reader after 24 h incubation at 37 ℃ in anaerobic condition. Triplicated results are expressed as the mean (%) ± SEM compared to the each control. Statistical analysis was conducted using two-way analysis of variance (ANOVA) with Bonferroni test. (*p<0.05, **p<0.01, ***p<0.001) SNUG10271, KCTC3018; *L. plantarum*. KCTC3019; *L. casei*, SNUG50057, SNUG50070; *L. rhamnosus*.

## A: *C. difficile* KCTC 5009




## B: *C. difficile* ATCC 43255

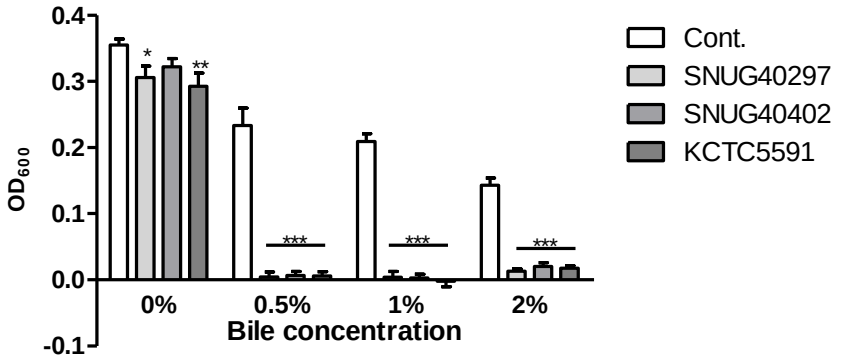



**Figure 5. Effects of bile mixture in culture supernatants of *C. scindens* isolates on *C. difficile* growth**

Inhibitory effect of *C. scindens* when it was cultured in bile mixture containing media. A) *C. difficile* KCTC 5009 growth OD; B) *C. difficile* ATCC 43255 growth. $OD_{600}$ values were measured by microplate reader after 24 h incubation at 37 ℃ in anaerobic condition. Triplicated results are expressed as the mean (%) ± SEM compared to the each control. Statistical analysis was conducted using two-way analysis of variance (ANOVA) with Bonferroni test. (*p<0.05, **p<0.01, ***p<0.001)

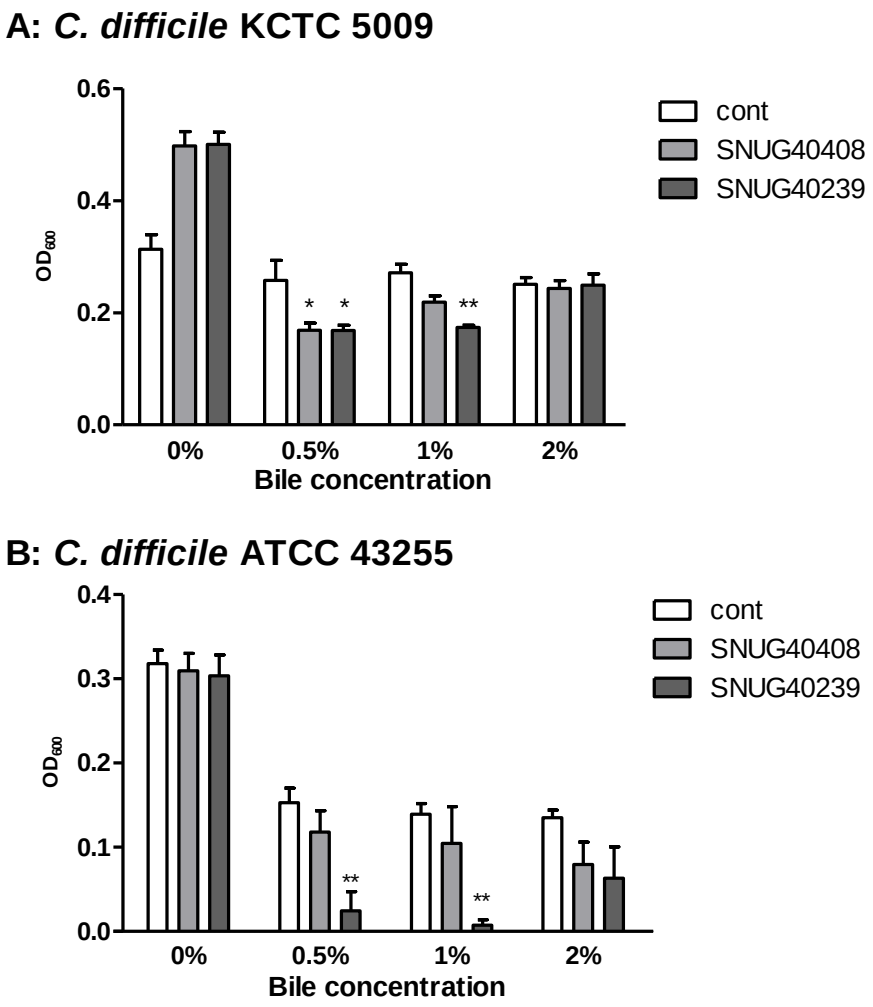



**Figure 6. Effects of bile mixture in culture supernatants of *B. ovatus* isolates on *C. difficile* growth**

Inhibitory effect of *B. ovatus* when it was cultured in bile mixture containing media. The supernatants were neutralized with NaOH and HCL to pH 6.7 ± 0.2. A) *C. difficile* KCTC 5009 growth OD; B) *C. difficile* ATCC 43255 growth. $OD_{600}$ values were measured by microplate reader after 24 h incubation at 37 ℃ in anaerobic condition. Triplicated results are expressed as the mean (%) ± SEM compared to the each control. Statistical analysis was conducted using two-way analysis of variance (ANOVA) with Bonferroni test. (*p<0.05, **p<0.01, ***p<0.001)

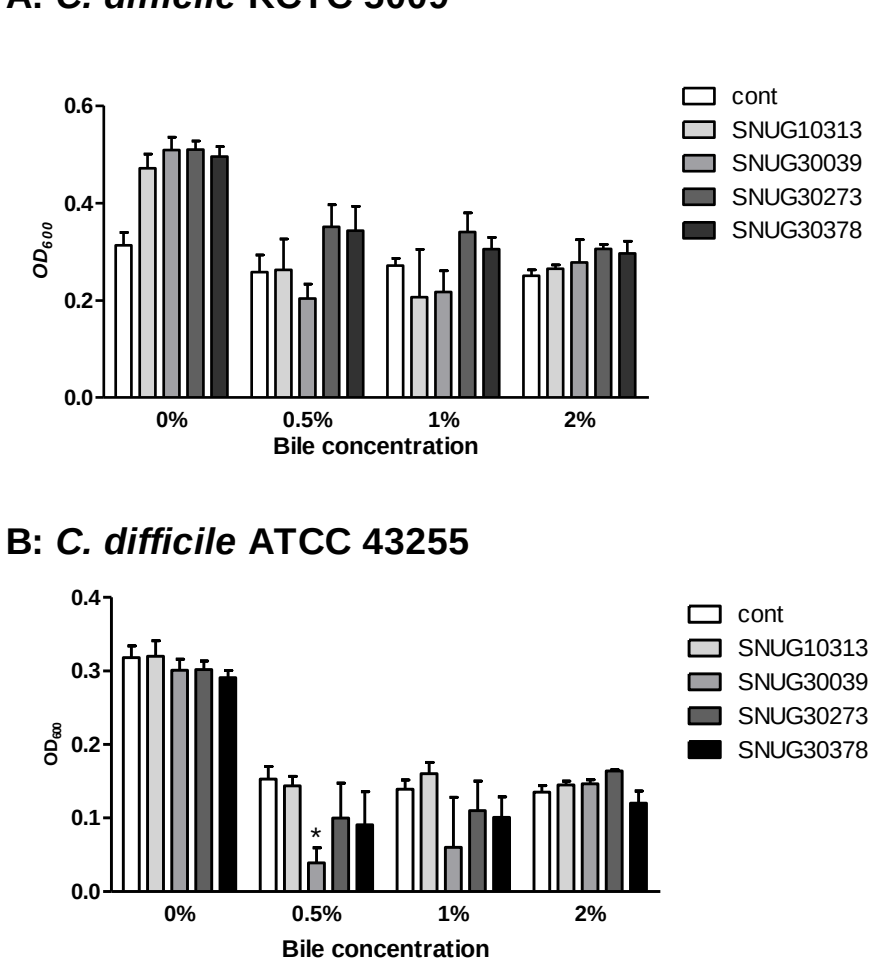



**Figure 7. Effects of bile mixture in culture supernatants of *B. caccae* isolates on *C. difficile* growth**

Inhibitory effect of *B. caccae* when it was cultured in bile mixture containing media. The supernatants were neutralized with NaOH and HCL to pH 6.7 ± 0.2 A) *C. difficile* KCTC 5009 growth OD; B) *C. difficile* ATCC 43255 growth. $OD_{600}$ values were measured by microplate reader after 24 h incubation at 37 ℃ in anaerobic condition. Triplicated results are expressed as the mean (%) ± SEM compared to the each control. Statistical analysis was conducted using two-way analysis of variance (ANOVA) with Bonferroni test. (*p<0.05, **p<0.01, ***p<0.001)

## 4. Butyrate, or bacterial supernatant have no observational effect to attenuate cytotoxicity

Toxin A and Toxin B, major virulence factors of *C. difficile*, enter the host cell via endocytosis, and then active domain is cleaved and released into the cytosol. This active domain results in host GTPases glucosylation, and furthermore, cytoskeleton is disrupted and cell rounding is observed microscopically. To evaluate whether gut isolates can prevent cytotoxic effect of *C. difficile*, bacterial culture supernatants were added into pre-incubated Caco2 and HT29 cell with toxin-containing supernatant of *C. difficile*.

Traditionally, *C. difficile* ATCC 43255 was thought to be hyper-toxin producing strain, frequently used in cytotoxicity test (12, 13, 36, 37), whereas KCTC 5009 was not. Although *C. diffcile* KCTC 5009 is also positively have *tcdA* and *tcdB* toxin gene (38), we can confirm that toxicity of ATCC 43255 is higher than KCTC 5009 (Figure 8). Supernatant of ATCC 43255 was used in further test for evaluation of bacterial isolates.

24 h after toxic supernatant dilutions were added, any of bacterial isolates show preventive effect when they added with *C. difficile* supernatant in cytotoxicity assay. Only *F. prausnitzii* SNUG30093 and *R. intestinalis* SNUG30016 were looks slightly better than control at toxic dilution ratio 1:320, but no observational effect at more concentrated dilution (Figure 9). For HT29, any slight difference between control and isolates was not shown.

Because toxin A and B were known to be vulnerable in acidic condition, *Lactobacilus* spp. was expected to show decreased cytotoxicity. However,

though typical cell rounding effect of tcd toxins were not observed, Caco2 cells deformed appearance and low cell density, probably due to low pH conditions owing to acetic acid production (Table 3). This abnormality was disappeared, result from neutralizing supernatant, and cell rounding was restored. In addition, test with sodium butyrate (Sigma-Aldrich) (concentration range from 30mM to 3.75mM) was not effective (data not shown)

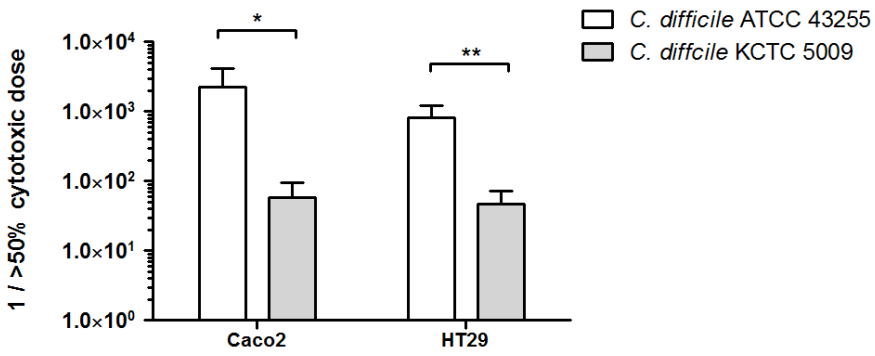



**Figure 8. Cytotoxic test results of *C. difficile* ATCC 463255 and KCTC 5009**

*C. difficile* ATCC 43255 and KCTC 5009 were cultured in TY broth for 24 h in anaerobic condition, and then culture supernatants were harvested. 4-fold dilutions of toxin-containing supernatants were added into pre-incubated Caco2 and HT29 cell. >50% cytotoxic doses were assessed microscopically after 24 h incubation. At least duplicated results are expressed as the mean (%) ± SEM. Statistical analysis was conducted using student's t-test (*$p<0.05$, **$p<0.01$)

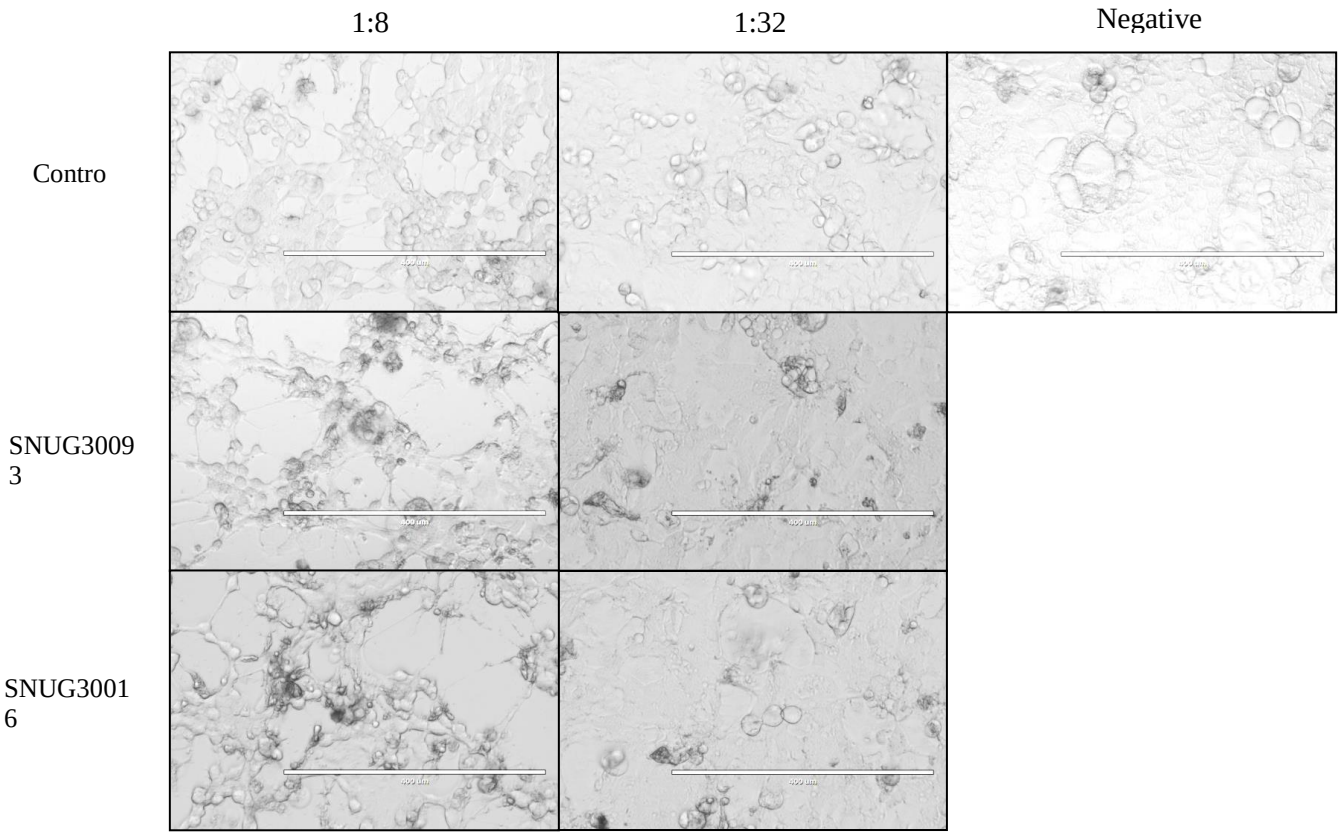



**Figure 9. Images of cytotoxicity test with bacterial supernatant**

Caco2 cells were seeded in 96 well plate, and after 2day incubation, each isolate's supernatants and *C. difficile* supernatant dilutions (ratios from 1:20, 1:80, 1:320, 1:1280 to 1:5120) were added. Cell rounding was assessed by microscopically. Negative; *C. difficile* supernatant were replaced with PBS.

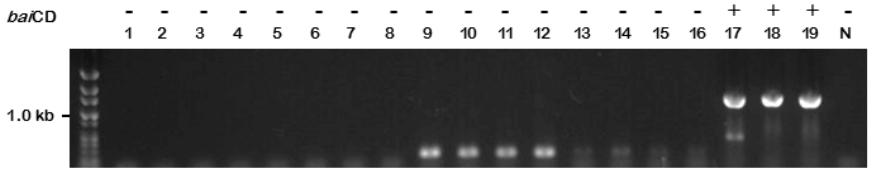
**Table 3**. SCFAs concentrations in bacterial supernatants(μmol/mL) and pH values

| Species | Strains | Acetate | Propionate | i-Butyrate | n-Butyrate | i-valerate | n-valerate | pH |
|---|---|---|---|---|---|---|---|---|
| *Bacteroides caccae* | | | | | | | | |
| | SNUG10313 | nd | nd | nd | nd | nd | nd | 6.28 ± 0.007 |
| | SNUG30039 | nd | nd | nd | nd | 0.93 | nd | 5.74 ± 0.007 |
| | SNUG30273 | nd | nd | nd | nd | 1.35 | nd | 5.79 ± 0.035 |
| | SNUG30378 | 7.52 | nd | nd | nd | 1.18 | nd | 5.68 ± 0.007 |
| *Bacteroides ovatus* | | | | | | | | |
| | SNUG40408 | 8.35 | 1.25 | 0.50 | nd | 0.44 | nd | 5.56 ± 0.021 |
| | SNUG40239 | nd | nd | nd | nd | 1.17 | nd | 5.58 ± 0.014 |
| *Clostridium scindens* | | | | | | | | |
| | SNUG40297 | 16.50 | nd | nd | nd | nd | nd | 5.48 ± 0.007 |
| | SNUG40402 | 15.95 | nd | nd | nd | nd | nd | 5.71 ± 0.000 |
| | KCTC5591 | 11.37 | nd | nd | nd | nd | nd | 6.03 ± 0.007 |
| *Faecalibacterium prausnitzii* | | | | | | | | |
| | SNUG30079 | nd | nd | nd | 4.63 | nd | nd | 6.80 ± 0.014 |
| | SNUG30106 | nd | nd | nd | 3.57 | nd | nd | 6.96 ± 0.021 |
| | SNUG30120 | nd | nd | nd | 4.34 | nd | nd | 6.85 ± 0.000 |
| | SNUG30093 | nd | nd | nd | 8.45 | nd | nd | 6.12 ± 0.007 |
| *Roseburia intestinalis* | | | | | | | | |
| | SNUG30016 | nd | nd | nd | 14.05 | nd | nd | 6.25 ± 0.014 |
| *Lactobacillus plantarum subsp. plantarum* | | | | | | | | |
| | SNUG10271 | 35.51 | nd | nd | nd | nd | nd | 4.32 ± 0.007 |
| | KCTC3018 | 32.63 | nd | nd | nd | nd | nd | 4.31 ± 0.000 |
| *Lactobacillus casei* | | | | | | | nd | |
| | KCTC3019 | 33.07 | nd | nd | nd | nd | nd | 4.13 ± 0.007 |
| *Lactobacillus rhamnosus* | | | | | | | | |
| | SNUG50057 | 32.13 | nd | nd | nd | nd | nd | 4.25 ± 0.170 |
| | SNUG50070 | 32.73 | nd | nd | nd | nd | nd | 4.43 ± 0.014 |

nd: not detected

## 5. Confirmation of *bai*CD gene of *C. scindens*

There are several pathways that metabolize bile acid. Above all, 7α-dehydroxylation is pointed out as a factor which correlated with CDI in several studies (9, 39, 40). The responsible enzyme is encoded in *bai*CD (bile acid inducible operon genes). Some tested isolates reduced *C. difficile* growth only when they were cultured with bile mixture, so we performed PCR assay of *baiCD* to know whether these effects result from 7α-dehydroxylation. Although some of *Bacteroides* were assumed to metabolize bile acid, all tested intestinal isolates were negative except *C. scindens* (Figure 10).




| | | | | | |
|---|---|---|---|---|---|
| 1 | *Faecalibacterium prausnitzii* | SNUG30079 | 11 | *Bacteroides caccae* | SNUG30273 |
| 2 | *Faecalibacterium prausnitzii* | SNUG30079 | 12 | *Bacteroides caccae* | SNUG30378 |
| 3 | *Faecalibacterium prausnitzii* | SNUG30092 | 13 | *Bacteroides ovatus* | SNUG40408 |
| 4 | *Faecalibacterium prausnitzii* | SNUG30106 | 14 | *Bacteroides ovatus* | SNUG40408 |
| 5 | *Faecalibacterium prausnitzii* | SNUG30120 | 15 | *Bacteroides ovatus* | SNUG40239 |
| 6 | *Faecalibacterium prausnitzii* | SNUG30093 | 16 | *Bacteroides ovatus* | SNUG40239 |
| 7 | *Faecalibacterium prausnitzii* | SNUG30090 | 17 | *Clostridium scindens* | SNUG40297 |
| 8 | *Roseburia intestinalis* | SNUG30016 | 18 | *Clostridium scindens* | SNUG40402 |
| 9 | *Bacteroides caccae* | SNUG10313 | 19 | *Clostridium scindens* | KCTC5591(ATCC35704) |
| 10 | *Bacteroides caccae* | SNUG30039 | N | DW | |

**Figure 10. PCR result with *bai*CD specific primers**

PCR result with *bai*CD specific primers. All tested intestinal isolates were negative except *C. scindens*. *C. scindens* KCTC5591 was used as positive control. PCR products were sequenced and identified as *bai*CD region via BLASTn. Vague band located below of the image, PCR products of *Bacteroides*, were identified as irrelevant to *bai*CD (query results were partial sequence of Lysine—tRNA ligase)

### 6. *C. difficile* vegetative cell growth with various bile acids

In the vegetative cell growth inhibition test, *C. difficile* ATCC43255 and KCTC5009 shows different susceptibility against bile acid mixture. To figure out whether it was caused by some specific bile acid, two strains were inoculated in broth media supplemented with various bile acids. Growth patterns of two strains were almost same, but different when they grew with CA (cholic acid). ATCC43255 growth was inhibited by 0.1% CA, whereas KCTC5009 was almost not.

Secondary bile acid, LCA and DCA inhibit *C. difficile* growth, like previously described (9), but the inhibitory effect of LCA was overcome as time passes. CDCA, one of primary bile acid but known as *C. difficile* germination and vegetative cell growth inhibitor (47, 48), shown vegetative cell growth inhibition at the concentration of 0.01%.

Other primary bile acids except CA and CDCA, have no effect to *C. difficile* KCTC5009 vegetative cell growth, but *C. difficile* ATCC43255 was slightly reduced.

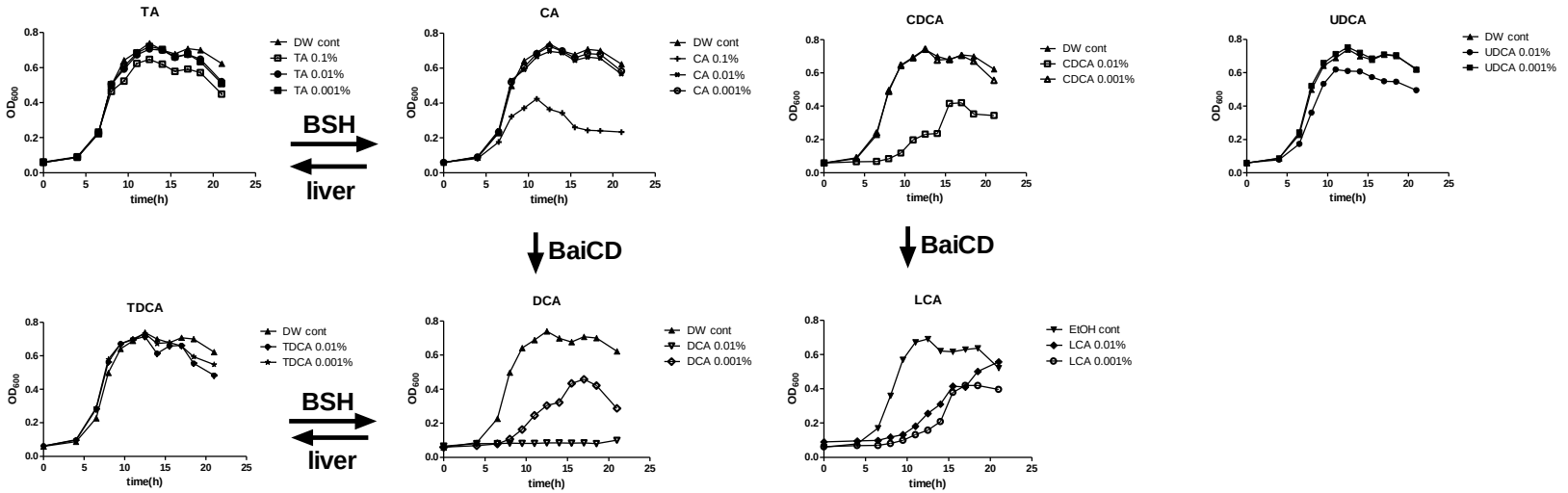



**Figure 2. *C. difficile* ATCC43255 growth with bile acids**

*C. difficile* ATCC43255 was inoculated 1% in BHIS supplemented with bile acids (Sigma-Aldrich). Each vehicle for bile acids (DW and 50% EtOH) were used for control.

(UDCA: Ursodeoxycholic acid, LCA: Lithocholic acid, CDCA: Chenodeoxycholic acid, DCA: Deoxycholic acid, TDCA: Taurodeoxycholic acid, CA: cholic acid, TA: Taurocholic acid)

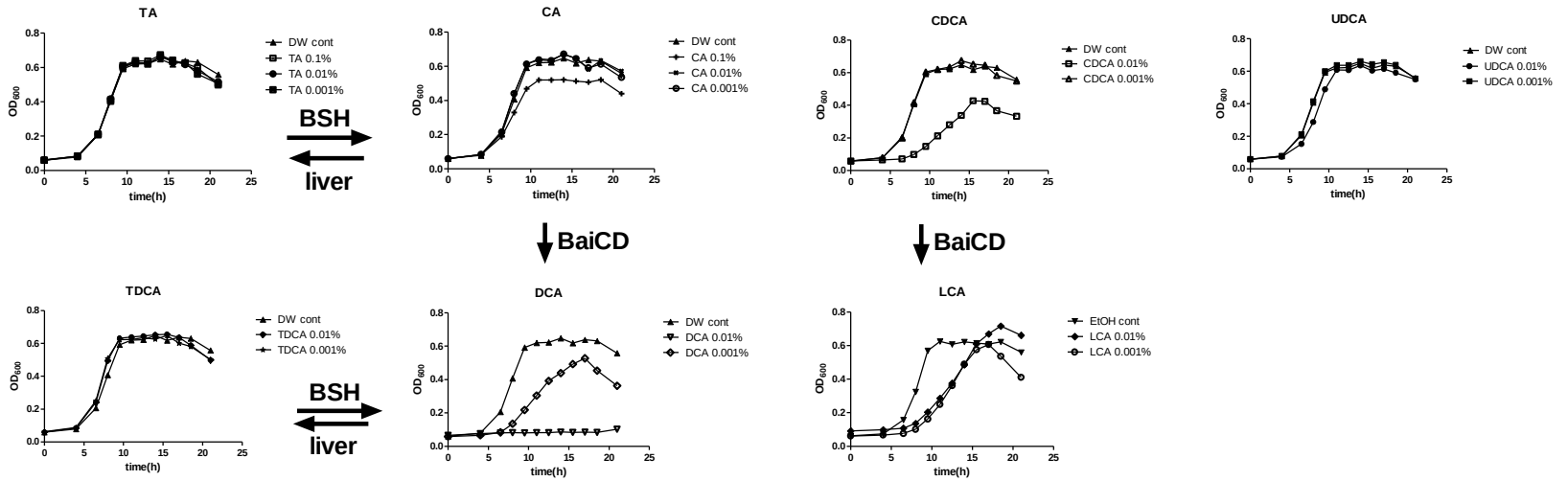



**Figure 3. *C. difficile* KCTC5009 growth with bile acids**

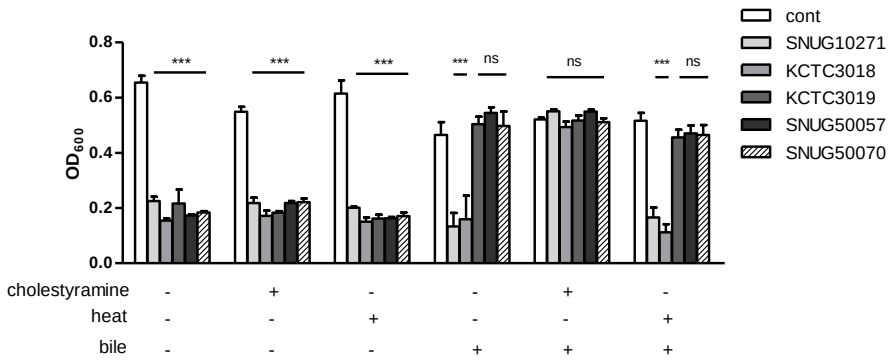
*C. difficile* KCTC5009 was inoculated 1% in BHIS supplemented with bile acids (Sigma-Aldrich). Each vehicle for bile acids (DW and 50% EtOH) were used for control.

(UDCA: Ursodeoxycholic acid, LCA: Lithocholic acid, CDCA: Chenodeoxycholic acid, DCA: Deoxycholic acid, TDCA: Taurodeoxycholic acid, CA: cholic acid, TA: Taurocholic acid)

## 7. Effects of cholestyramine and heat treatment

The Correlation of *baiCD* with CDI was already well-known, but *in vitro* or *in vivo* information of *bsh* against CDI is limited to *in silico* data, currently (9, 45). To figure out inhibition of vegetative cell growth of *C. difficile* when it was tested with bile-containing bacterial supernatants, especially supernatants of *baiCD*-negative bacteria like *Lactobacillus* and *Bacteroides ovatus*, cholestyramine and heat treatment was done to supernatant. Cholestyramine was used to treat hypercholesterolemia before the advent of statins, because it absorbs bile acid. After treatment of cholestyramine, bile-containing supernatants were lost their effectiveness, and their effectiveness was not affected by heat treatment (Figure 13, 14).

## A: *C. difficile* KCTC 5009




## B: *C. difficile* ATCC 43255

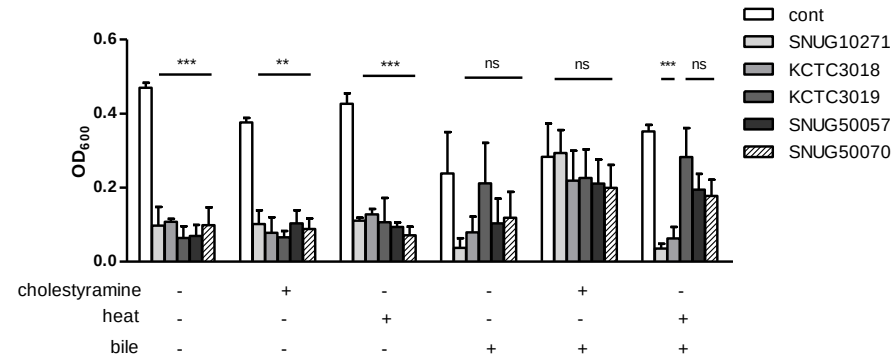
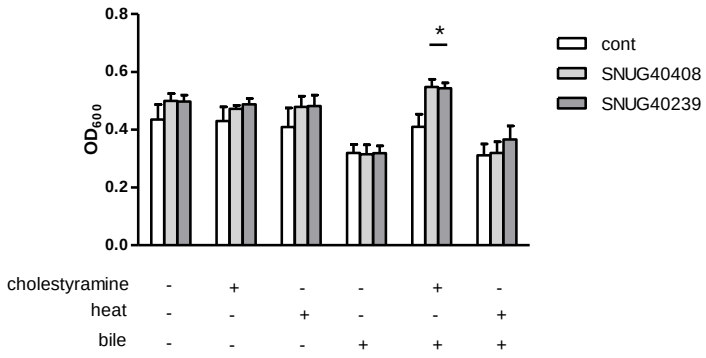



**Figure 4. Effects of cholestyramine and heat treatment on culture supernatants of *Lactobacillus* isolates on *C. difficile* growth**

After neutralization, bile 0.5% supernatants were treated with cholestyramine resin(50 mg/ml, 60min with vortex) or heat (100 ℃, 60min). A) *C. difficile* KCTC 5009 growth OD; B) *C. difficile* ATCC 43255 growth. $OD_{600}$ values were measured by microplate reader after 24 h incubation at 37 ℃ in anaerobic condition. Triplicated results are expressed as the mean (%) ± SEM compared to the each control. Statistical analysis was conducted using two-way analysis of variance (ANOVA) with Bonferroni test. (**p<0.01, ***p<0.001)

## A: *C. difficile* KCTC 5009




## B: *C. difficile* ATCC 43255

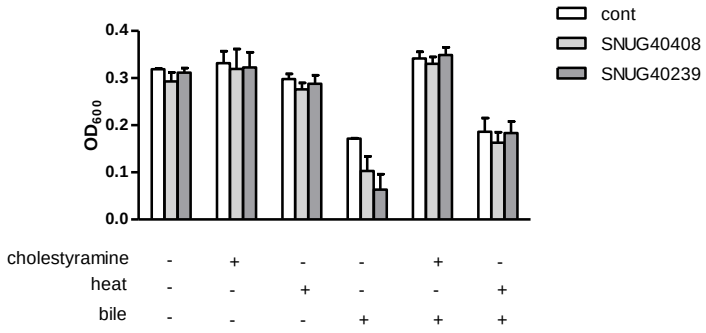



**Figure 5. Effects of cholestyramine and heat treatment on culture supernatants of *B. ovatus* isolates on *C. difficile* growth**

After neutralization, bile 0.5% supernatants were treated with cholestyramine resin(50 mg/ml, 60min with vortex) or heat (100 ℃, 60min). A) *C. difficile* KCTC 5009 growth OD; B) *C. difficile* ATCC 43255 growth. $OD_{600}$ values were measured by microplate reader after 24 h incubation at 37 ℃ in anaerobic condition. Triplicated results are expressed as the mean (%) ± SEM compared to the each control. Statistical analysis was conducted using two-way analysis of variance (ANOVA) with Bonferroni test. All results were not significant.

# IV. Discussion

Primary bile acids, especially, cholic acid derivatives (glycocholic acid, taurocholic acid) are essential for *C. difficile* spore germination. On the other hand, secondary bile acids, entirely produced by gut commensal bacteria, shows inhibitory effect for both vegetative cell growth and spore germination. Recently, Weingarden AR et al. showed that the changes in fecal bile acid composition by FMT (fecal microbiota transplantation) prevent *C. difficile* to germinate; in post-FMT, secondary bile acid DCA (deoxycholic acid) and LCA (lithocholic acid) concentration was increased, and primary bile acid TA, CA and CDCA (chenodeoxycholic acid) concentration was decreased, result in inhibition of *C. difficile* spore germination (14, 15).

There are two important bacterial genetic elements responsible for bile acid metabolism, *bsh* (bile salt hydrolase gene) and *bai* (bile acid inducible) operon. BSH deconjugate bile acids, whereas *bai*CD takes charge in 7α-dehydroxylation. Buffie et al. showed BSH was not correlated with CDI significantly, while *bai* operon was significantly correlated with CDI (9). However, result of our *in vitro* evaluation, some of isolates shows more effective when they were grown with bile mixture even though they don't have *bai*CD gene (Figure 10), probably result from some other bile salt metabolizing enzyme, like *bsh*. Otherwise, existence of bile mixture in culture medium has some synergistic effect, such as stimulating expression of inhibitory molecules like bacteriocin. Recently, Allegretti et al. found significant association between *bsh* and CDI (45). This finding gives more

weight to the assumption that *bsh* is the cause of inhibitory activity of *Lactobacillus*, known as potent *bsh* producer.

CA (cholic acid), one of primary bile acid, is known as *C. difficile* germinant. However, in this study, it shows notable inhibition for *C. difficile* ATCC43255 vegetative cell growth at the concentration 0.1%. Conjugated cholic acids like TA (taurocholic acid) could be deconjugated by BSH and changed into CA. Thus, *bsh* positive bacteria could inhibit *C. difficile* ATCC43255 more selectively than *C. difficile* KCTC5009. *C. difficile* inter-strain variation of germinant receptor is correlated with their response to bile acids (15). In this way, vegetative cell growth could be affected by bile acid receptor genetic variation of *C. difficile*.

All of *Bacteroides* isolates didn't show significant decrease on *C. difficile* growth without bile mixture, but they show inhibitory activity from agar spot assay (Figure 1, 3 and Table2). In agar spot assay, bacterial colonies were remained on agar surface. Therefore, the cells of isolates itself or interaction of two species when they grow on together could be the reason of inhibitory effect of agar spot assay. However, because *C. difficile* is sensitive to acid condition, lowered pH due to SCFA producing, particularly isovaleric acid for *Bacteroides*, could be the reason.

*F. prausnitzii* and *R. intestinalis* didn't have activity at any concentration, whereas *Lactobacillus* reduced *C. difficile* growth regardless of bile existence. The former two species were vulnerable to bile, but the latter was not. Also, *B.*

*caccae* isolates SNUG 30039, and *B. ovatus* isolates showed better growth in media with bile mixture than the other *B. caccae*. This result implying bile resistance may act as indication for inhibitory activity. In addition, *Lactobacillus* isolates also show good growth with 4% bile acid, and they are known for having *bsh* gene (23).

Timothy D. H. Woo et al. reported that *C. difficile* VPI 10463 growth was reduced at pH under 5.5 (16). L.J. Wheeldon et al. showed that pH affects spore germination, thus higher gastric pH may be favored by *C. difficile* (17). Before neutralizing supernatant, Lactobacillus greatly dropped *C. difficile* growth. However, not like in laboratory condition, colon contents have higher pH values even if *Lactobacillus* is colonized. Thus, all bacterial supernatants were neutralized for realistic evaluation.

Butyrate, one of major metabolite result from bacterial fermentation of dietary fiber, has been thought to be beneficial because of their anti-inflammatory effect in human intestine (18). Nevertheless, supernatants and butyrate solution were failed to prevent cytotoxicity. Because microscopic observation is not sufficiently objective and sensitive, more sensitive methods needed to measure slight effect. However, since *C. difficile* also produce butyrate, this ineffectiveness of butyrate is not surprising. Furthermore, because butyrate promotes toxin production of *C. difficile* (19), it could aggravate symptoms of CDI.

In conclusion, butyrate-producing two species, *F. prausnitzii* and *R. intestinalis* are not a good option for CDI. In spite of response to bile acid were different between *C. difficile* strains, bile acid metabolizing bacteria, including *Bacteroides*, could be effective. *C. scindens* was chosen in this study because of their 7α-dehydroxylae activity, but they showed reduced growth of *C. difficile* KCTC5009 without bile mixture. This indicates *C. scindens* inhibit *C. difficile* growth in a different manner, such as nutrient depletion or production of bacteriocin. Because *C. scindens* and *C. difficile* are same *Clostridium* genus, they have some common metabolic characteristics like sialic acid metabolism (22).

Interestingly, hyper toxin producing strain *C. difficile* 43255, also known as low sporulation strain, shows lower bile resistance than *C. difficile* KCTC 5009, especially against for CA (Figure 4-7, 11-12). It is well known fact that *C. difficile* sporulation is inhibited in toxin-producing environmental conditions, because of co-regulation of virulence genes (34, 35). Elucidated environmental signals that regulate *C. difficile* toxin gene expressions are butyrate (up-regulate toxin producing), glucose and some amino acids (proline, cysteine). In addition that, these different bile acid sensitivity between two strains of *C. difficile*, one is hyper toxigenic and another is lower toxigenic, may imply bile acids have some another role in regulation of virulence factors. Besides, *F. prausnitzii* and *R. intestinalis* show reversed inhibitory pattern on *C. difficile* growth (Figure 1). This observation could be another example of co-regulation, because the more they produced butyrate, the more contrast

was made.

Colonization of *C. difficile* is complicatedly regulated by various metabolites. Although sporulation play an important role in CDI recurrence and transmission, lowering sporulation could result in elevated toxin level thus deteriorate acute symptoms (35). For searching appropriate candidate for developing bacteriotherapeutics, multilateral characteristics should be evaluated under the extensive understanding of regulatory features of *C. difficile*.

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국문초록

# 한국인 장내유래 세균을 대상으로 한 *Clostridium difficile*의 저해 효과 연구

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*Clostridium difficile* 감염 (이하 CDI; *Clostridium difficile* infection)은 대표적인 원내감염의 하나로써, 항생제 사용으로 인한 장내 정상 세균총의 불균형과 관련이 있는 것으로 알려져 있다. 그러나 어떤 종의 세균이 그러한 역할을 하는지, 혹은 어떠한 기전으로 CDI에 대한 저항성이 발현되는지에 대해서는 아직까지 연구되어야 할 부분이 많다. 밝혀진 바에 의하면 세균에 의한 대사물질이 CDI 저항성을 매개하는 사례들이 있다. Taurocholate를 비롯한 일차담즙은 *C. difficile* 포자의 발아를 촉진하는 역할을 하는 반면, 장내 세균의 효소에 의해 대사되어 생성되는 이차담즙은 *C. difficile* 균의 성장 및 포자의 발아를 저해한다. 또한 butyrate를 비롯한 단쇄지방산은 장내 염증 완화 효과를 가지는 것으로 알려져 있다. 본 연구에서는 한국인에게서 분리한 세균 중 담즙산의 대사하는 균과 단쇄지방산을 생성하는 균을 대상으로 *C. difficile*의 성장 저해 및 *C. difficile*의 독소로 인한 세포독성의 완화 효과가 있는지 알아보았다. 담즙을 대사하는 것으로 알려진 *Clostridium scindens, Lactobacillus, Bacteroides*는 중 담즙을 대사함으로 보다 강한 저해를 나타내는 것으로 추정되는 분리균주가 있었다. 단쇄지방산을 생성하는 세균의 상층액을 이용한 실험에서 세포독성의 저하는 관찰되지 않았으며, butyrate를 단독으로 사용한 실험에서도 마찬가지로 세포독성에 있어 변화는 관찰되지 않았다. 본 실험의 결과를 통해 담즙의 대사 능력이 *C. difficile*의 저해 균주 선발에 있어서 중요한 요소임을 재확인 할 수 있었다.

**주요 단어:** CDI, *Clostridium difficile*, 원내감염, 담즙, 단쇄지방산

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